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Selective cytotoxic eremophilane-type sesquiterpenes from *Penicillium citreonigrum*

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Abstract

One new eremophilane-type sesquiterpene (**1**, citreopenin) was isolated from *Penicillium citreonigrum* (HQ738282), and the structure was elucidated by a combination of spectroscopic data interpretation and single-crystal X-ray diffraction analysis using Cu Kα radiation (CCDC 1030588). Compound **1** showed weak activity against KB-VIN (IC_{50} = 11.0 ± 0.156 μM), while the known compound **3** exhibited selective cytotoxicity against MDA-MB-231 triple-negative breast cancer (TNBC) (IC_{50} = 5.42 ± 0.167 μM).

Keywords

Metabolites; *Penicillium citreonigrum*; eremophilane-type sesquiterpene; cytotoxic

1. Introduction

In our earlier study, two 11-noreremophilane-type sesquiterpenes (**3**, **4**) with a conjugated enolic OH group were identified from a culture of *Penicillium citreonigrum*, which was isolated from normal *Dryopteris setosa* (Thunb.) leaves [1]. Subsequent research indicated that the brominated compound **3** showed selective activity against the MDA-MB-231 triple-negative breast cancer (TNBC) cell lines. Based on this promising result, we continued isolation, structure elucidation, and activity evaluation of metabolites from *P. citreonigrum* in order to discover additional new bioactive compounds showing unique antitumor effects. We now have obtained a new eremophilane-type sesquiterpene, eremophila-1(10),3,7(11),8-

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tetraene-3-hydroxy-2-oxo-12,8-lactam (**1**), as well as the known mycophenolic acid (**2**) [2] (Figure 1).

The structures of the metabolites, **1** and **2**, were elucidated by a combination of spectroscopic data interpretation and single-crystal X-ray diffraction analysis. We also investigated the antiproliferative activities of **1**, **3**, and **4** against five human tumor cell lines, including A549 (lung carcinoma), MCF-7 (estrogen receptor positive, HER2-negative breast cancer), MDA-MB-231 (TNBC), KB (originally isolated from epidermoid carcinoma of the nasopharynx) and KB-VIN (P-glycoprotein-overexpressing vincristine-resistant KB subline showing MDR phenotype).

2. Results and discussion

Compound **1** was obtained as yellow crystals and its molecular formula was assigned as $C_{15}H_{15}NO_3$ on the basis of ^{13}C NMR (Table 1) and LCMS-IT-TOF data at *m/z* 256.0971 $[M-H]^-$ implying nine degrees of unsaturation. ^{13}C NMR and HMQC experiments revealed 15 well-resolved signals, including three CH_3, one CH_2 and two CH groups, and nine quaternary C-atoms.

The structure of **1** was identified by comprehensive analysis of 2D-NMR data, including results of ^1H,^1H-COSY, HMQC, and HMBC spectra (Table 1). The HMBC correlations [δ(H) 1.34 (14-Me)/*δ*(C) 131.4 (4), 43.0 (5), 31.7 (6), 162.6 (10); *δ*(H) 2.07 (15-Me)/*δ*(C) 144.8 (3), 131.4 (4), 43.0 (5); *δ*(H) 6.35 (1)/*δ*(C) 144.8 (3), 43.0 (5), 107.0 (9); *δ*(H) 6.15 (9)/*δ*(C) 123.2 (1), 43.0 (5), 138.7 (7); *δ*(H) 1.99 (13)/*δ*(C) 138.7 (7), 129.8 (11), 173.2 (12); *δ*(H) 3.05 (6*β*)/*δ*(C) 43.0 (5), 138.7 (7), 141.5 (8), 162.6 (10), 129.8 (11), 29.6 (14)] confirmed the presence of a eremophilane-type sesquiterpene skeleton (Table 1 and Figure 1).

Based on the molecular formula, the structure of **1** must contain a third ring to match the nine degrees of unsaturation. Furthermore, there are two active proton signals at 8.47 ppm (brs.) and 6.64 ppm (s) in the ^1H NMR spectrum (Figure S1). Most likely, the third ring is 12,8-lactam, and the group at C-3 is hydroxyl. Spectroscopic comparison indicated that compound **1** has the same skeleton as eremophila-1(10),7(11),8-triene-12,8 -lactam, which was isolated from *Ligularia fischeri* [3]. Thus, the planar structure of **1** was determined as shown in Figure 2 and was confirmed by X-ray diffraction analysis using Cu Kα radiation (CCDC 1030588), which also established the absolute configuration as 5*S* (Figure 2). Compound **1** (provisionally named citreopenin) was fully determined as eremophila-1(10), 3,7(11),8-tetraene-3-hydroxy-2-oxo-12,8-lactam.

From the ID selective NOESY correlation between *δ*(H) 1.34/*δ*(H) 3.05, the configuration of the H-6 proton at 3.05 ppm) was assigned as *β* (Figure 3). Therefore, the configuration of the H-6 proton at 2.40 ppm is *α*, and this proton was also correlated with Me-13 (1.99 ppm) in the COSY spectrum.

Compound **2** was determined as mycophenolic acid based on the comparison of experimental NMR data (Figures S8–S12) with reported data for mycophenolic acid [2].

The antiproliferative activities of **1**, **3**, and **4** against human tumor cell lines including A549, MDA-MB-231, KB, KB-VIN, and MCF-7 were investigated (Table 2). The results revealed that **3** exhibited selective activity against the triple-negative breast cancer (TNBC) cell lines MDA-MB-231 (IC_{50} = 5.42 ± 0.167 μM), while nontoxic at 40 μM against the remaining cell lines was tested. Interestingly, **3** induced cell cycle arrest at G2/M phase in MDA-MB-231 cells (Figure 4), suggesting that **3** targets mitosis controlling protein(s) expressed selectively in MDA-MB-231. Additionally, we found that **3** selectively inhibited tumor growth of P-glycoprotein (P-gp) overexpressing multidrug resistant (MDR) cell line KB-VIN (IC_{50} = 11.0 ± 0.156 μM). We speculated that the long conjugated system and halogen may contribute to these unique selective antiproliferative activities.

3. Experimental

3.1. General experimental procedures

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected (Thermo Fisher Scientific, Waltham, MA, USA). Optical rotations were recorded on a JASCO P-2000 polarimeter (JASCO, Tokyo, Japan). X-ray diffraction data were recorded on a Bruker axs Smart Apex II (Bruker, Karlsruhe, Germany). IR data were recorded on a Bruker Tensor 27 (Bruker, Karlsruhe, Germany). UV data were recorded on a Persee TU-1950 UV-KIS Spectrophotometer (Persee, Beijing, China). NMR experiments were performed on a GMB400-inova400 (Varian, Palo Alto, CA, USA) in $CDC1_3$ using TMS as internal standard. The HR-ESI-MS were recorded on a LCMS-IT-TOF (Shimadzu, Kyoto, Japan). Column chromatography was performed on Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) and silica gel (65 × 250 mesh, Sorbent technologies, GA, USA). ODS-AQ-HG (12 nm, 50 μm, YMC, Kyoto, Japan). TLC analysis was carried out on silica gel GF254 plates (Merck, Nils Oldenburg, Germany).

3.2. Strain and cultivation

The fungal species was isolated from the normal *D. setosa* (Thunb.) leaves collected in July 2010 from Zhangjiajie, Hunan Province, China, and was identified as *P. citreonigrum* using DNA amplification and sequencing of the ITS-5.8S rDNA region. The sequenced data have been deposited with the GenBank (accession No. HQ738282). A voucher strain of this fungus was preserved at the Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, Jiangsu Normal University, Xuzhou, China. The fresh mycelia grown on PDA plates were inoculated into ten flasks (250 ml), each preloaded with 150 ml of autoclaved rice, followed by static culture at 28 °C for 90 d.

3.3. Extraction and isolation

The rice culture (10–150 ml) was extracted exhaustively with EtOH. The extracts were concentrated under reduced pressure to evaporate EtOH, and then the H_2O-suspended residue was extracted exhaustively with EtOAc. The EtOAc extract was concentrated under reduced pressure to give a brown oily residue (3.6 g), which afforded seven fractions, Frs. 1–7, upon CC (SiO_2; $CHCl_3$/MeOH 100:0 to 2:1). Fr. 5 (eluted with $CHCl_3$/MeOH, 10:1) was subjected to CC (ODS; MeOH/H_2O 60:40 to 100:0) to give **1** (8.5 mg) as light yellow

crystals. Fr. 3 (eluted with $CHCl_3$/MeOH 50:1) was separated by Sephadex LH 20 (100% MeOH) to give **2** (12 mg) as colorless crystals.

3.3.1. Eremophila-1(10),3,7(11),8-tetraene-3-hydroxyl-2-oxo-12,8-lactam (1)—

Yellow crystals; m.p. 197.6–199.4; $[\alpha]_D^{26.5}$ – 111.57 (c 0.778, $CHC1_3$); UV (MeOH) λmax: 206, 266, 294, 330, 363 nm. IR (KBr): 3186 (OH, NH), 2361, 2337 (CH), 1712 (C=0), 1679 (lactam C=0), 1642 (NH), 1612, 1595 (C=C), 1282 (=CH), 1138 (C–O), 909 (C–N) cm^{-1}; ^1H NMR ($CDC1_3$,400 MHz) and ^{13}C NMR ($CDC1_3$, 100 MHz) spectral data, see Table 1; ESI-MS (pos.): *mlz* 257.95 $[M+H]^+$, 280.05 $[M+Na]^+$, 296.20 ($[M+K]^+$); HR-ESI-MS (neg.): *mlz* 256.0971 $[M-H]^-$ (calcd for $C_{15}H_{14}N0_3$, 256.0974).

3.4. X-ray crystal structure determination

CCDC-1030588 contains the supplementary crystallographic data for compound **1**. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

3.5. Antiproliferative assay

Antiproliferative activities of compounds against five human tumor cell lines, including A549, MCF-7, MDA-MB-231, KB and KB-VIN, were evaluated by the sulforhodamine B assay after 72-h culture with compound as described in literature [4]. Paclitaxel was used as a reference drug. Cells were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with 2 mM L-glutamine and 25 mM HEPES (hydroxyethyl piperazineethanesulfonic acid, Corning, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA), 100 μg/ml streptomycin, 100 IU/ml penicillin, and 0.25 μg/ml amphotericin B (Mediatech/Corning, Manassas, VA, USA).

3.6. Cell cycle ahnalysis

Effects of **3** on cell cycle progression were evaluated by measurement of the cellular DNA content by propidium iodide (PI) (BD Biosciences) staining as described previously [4]. Compound **3** was used at 1, 10, or 40 μM. After a 24-h treatment of MDA-MB-231 cells, cells were fixed in 70% EtOH overnight at −20 °C, followed by staining with PI overnight at 4 °C. Stained cells were analyzed by flow cytometer (FACScalibur, BD Biosciences). Experiments were repeated three times.

Acknowledgments

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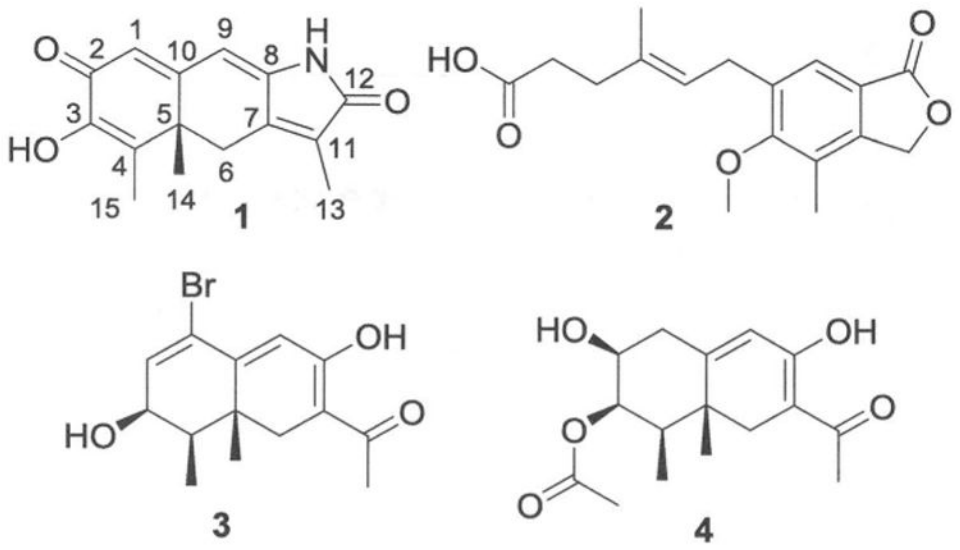

Figure 1.
Structures of compounds **1–4.**

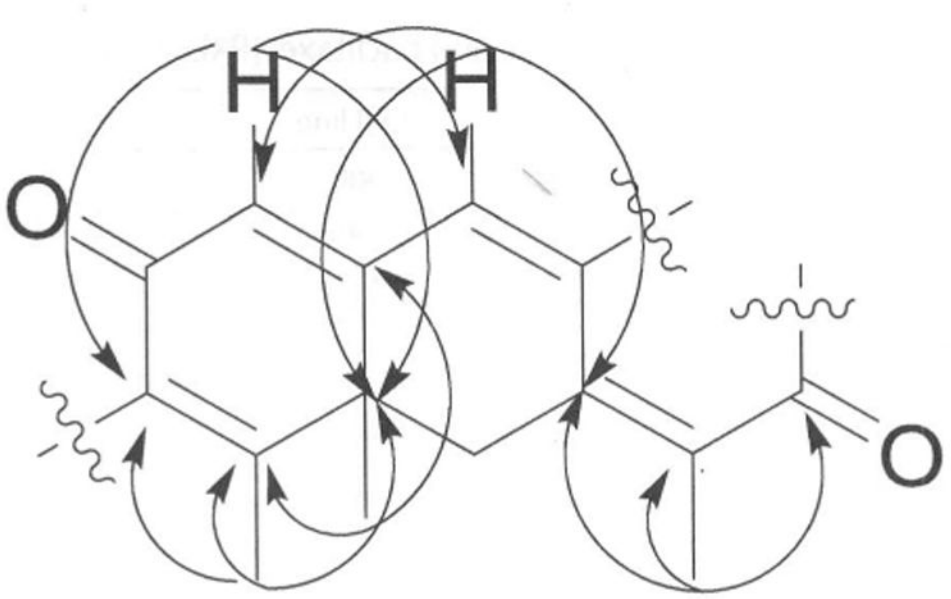

Figure 2.
Key HMBC correlations of **1**.

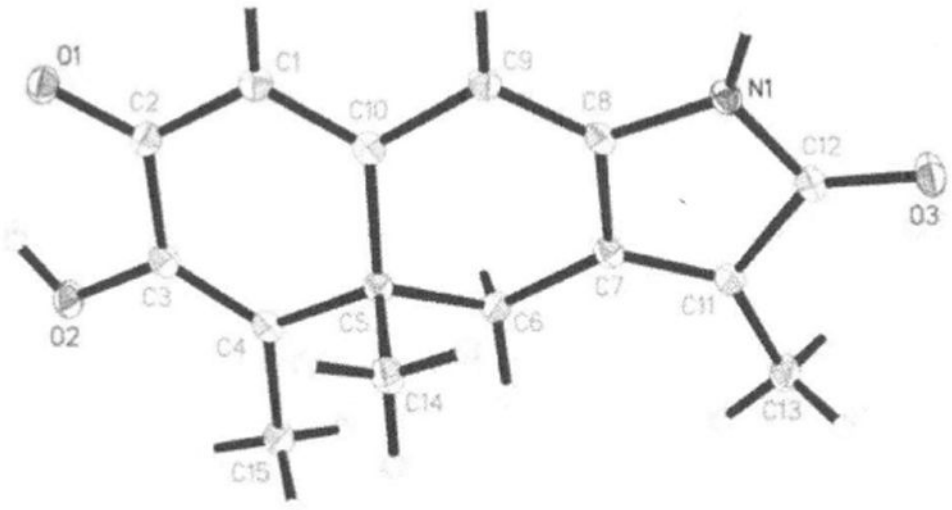

Figure 3.
X-ray single-crystal structure of **1**.

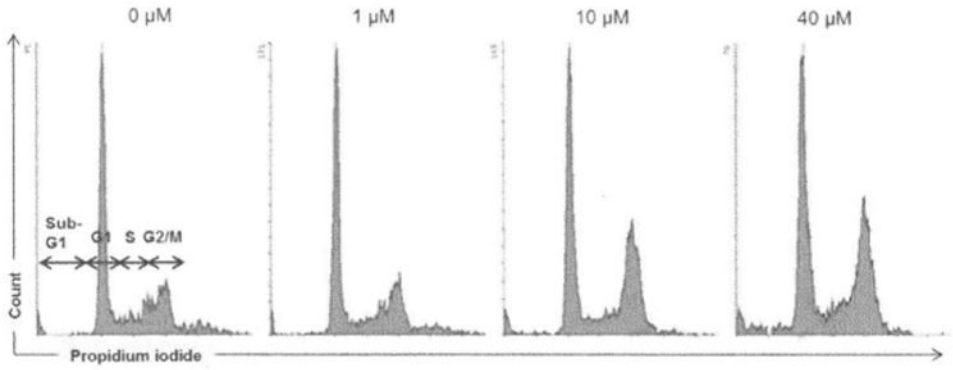

Figure 4.
Induction of cell cycle arrest at G2/M phase by 3 in TNBC cell line (MDA-MB-231).

Table 1

^{1}H-, ^{13}C-NMR spectroscopic data (400 and 100 MHz, resp.), ^{1}H-^{1}H COSY and HMBC correlations of 1 recorded in $CDCI_3$ (δ in ppm, J in Hz).

	δ(H)	δ(C)	^{1}H-^{1}H COSY	HMBC
1	6.35 *s*	123.2		C-3, 5, 9
2		179.6		
3		144.8		
4		131.4		
5		43.0		
6	β: 3.05 (*d*, *J*=16.0); α: 2.40 (*dd*, *J*= 16.0, 4.0)	31.7	H-13	C-5, 7, 8, 10, 11, 14
7		138.7		
8		141.5		
9	6.15 *s*	107.0		C-1, 5, 7
10		162.6		
11		129.8		
12		173.2		
13	1.99 (*d*, *J*=4.0)	8.8	6α-H	C-7, 11, 12
14	1.34 *s*	29.6		C-4, 5, 6, 10
15	2.07 *s*	11.2		C-3, 4, 5
NH	8.47 brs.			
OH	6.64 s			

Table 2

Antiproliferative data of **1**, **3**, **4** [IC_{50} (μM)], and paclitaxel [PXL, IC_{50} (nM)].

	Cell line				
Compound	**A549**	**MDA-MB-231**	**KB**	**KB-VIN**	**MCF-7**
1	>40	>40	>40	11.0 ± 0.16	>40
3	19.2 ± 0.40	5.41 ± 0.17	14.8 ± 0.45	18.8 ± 0.39	18.2 ± 1.52
4	>40	>40	>40	>40	>40
PXL	0.81 ± 0.09	6.06 ± 0.71	0.37 ± 0.05	2189.00 ± 100.43	9.13 ± 0.27